

## PROTEIN RIM2

### FIELD OF THE INVENTION

The present invention relates to protein Rim2, which is a novel isoform of Rim, i.e., a protein that interacts with a low molecular G protein Rab3 and is proposed to serve as a regulator of Rab3-dependent synaptic vesicle fusion, and which specifically interacts with the GDP/GTP exchange factor (GEFII; a cAMP sensor). More specifically, the present invention relates to elucidation of the mechanisms of intracellular vesicle transport and secretion, and to the novel protein Rim2 which is useful in diagnosis of endocrine-related diseases or neuropathy and in development of agents for prevention and treatment thereof, the gene encoding Rim2 and an antibody addressed to Rim2 protein.

Rim2 is considered to be a regulatory factor of vesicle fusion. It was found in the course of the present invention that the protein is expressed predominantly in endocrine tissues and endocrine- and neuroendocrine-derived cell lines. GTP-Rab3/GEFII/Rim complex is thought to participate in the regulation of exocytosis of neurons and endocrine cells, in a cAMP-dependent and protein kinase A (PKA) independent manner.

### BACKGROUND OF THE INVENTION

Transport of substances between cell organelles, which are unit membrane-enclosed structures such as endoplasmic reticulum, is conducted by intracellular vesicle transport. In endocrine cells including pancreatic  $\beta$ -cells and pituitary cells, peptides/proteins synthesized at ribosomes are received by the endoplasmic reticulum, from which they are transported in vesicles, which are transformed into secretory vesicles through the Golgi body and transported to the cell membrane, where they are released out of the cell via a step which includes fusion of the membranes. In neurons, neurotransmitter-containing precursors of synaptic vesicles are formed in Golgi bodies and transported by microtubules along the axon and stored at the synapse. Depolarization of the pre-synaptic membrane causes the vesicles to fuse with the pre-synaptic membrane and thus the neurotransmitters are released. This type of secretion based on the fusion of the vesicles and the cell membrane is called exocytosis.

In contrast, when extracellular substances such as hormones including cell

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growth factors are bound to the cell membrane, the complexes thus formed are invaginated into the cell to form endosomes. This type of uptake of environmental substances is called endocytosis.

5 Formation of vesicles, such as by budding, commonly observed both in exocytosis and endocytosis, and docking and fusion, the phenomena observed in process of their transportation and binding to other membrane systems, are regulated by a GTP-binding, low-molecular protein, called G protein. More than 30 types of this protein are known. The group of the proteins, which are also classified in Rab family, regulate the intracellular vesicle transport system.

10 With regard to the intracellular vesicle transport system, it is understood today that a cell is in a resting state when Rab protein occurs in a bound form to guanine nucleotide diphosphate (GDP), and that budding, docking and fusion are triggered as a result of a process in which a protein having GEF activity act on Rab protein and converts it to GTP-binding Rab protein, to which GTP binds to form a  
15 GTP-Rab complex, which in turn binds to a corresponding target protein on the membrane.

Stimulus-secretion coupling plays an important role in exocytosis observed in many cell types including neurons and endocrine cells [J.E. Rothman, Nature 372:55(1994); T.C. Sudhof, Nature 375:645 (1995)]. While a rise in intracellular  
20  $Ca^{2+}$  concentration is important in the regulation of exocytosis, other signals are also known to play important roles. cAMP (cyclic adenosine-3',5'-monophosphate)/PKA (cAMP-dependent protein kinase A) signaling pathway is known to regulate exocytosis in many of neurons, neuroendocrine cells and endocrine cells. In particular, cAMP has been thought to mediate long-term  
25 potentiation by increasing neurotransmitter release in the brain [R.D. Hawkins et al. Ann. Rev. Neurosci. 16:625(1993); G. Lonart et al., Neuron 21:1141(1998)]. cAMP also regulates exocytosis responsible for insulin release from pancreatic  $\beta$ -cells and amylase release from parotid acinar cells [P.M. Jones and S.J., Persaud, Endocrine. Rev. 19:429(1998); E. Renstrom, et al., J. Physiol. 502:105(1997); K.  
30 Yoshimura, Biochim. Biophys. Acta 1402:171(1998)].

In addition to its role in PKA-dependent phosphorylation of regulatory proteins associated with the process of exocytosis, it is known that cAMP also acts directly on the exocytotic machinery in neurons and non-neuronal cells [G. Lonart et al., Neuron 21:1141 (1998); E. Renstrom et al., J. Physiol. 502:105 (1997); K.

Yoshimura, *Biochim. Biophys. Acta*, 1402:171(1998)].

During the search by the yeast two-hybrid screen (i.e., a method for detection of the interaction between two proteins in yeast cells) for an intracellular signaling molecule directly coupling to a sulphonylurea receptor, a component of pancreatic  $\beta$ -cell ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels [N. Inagaki et al. *Proc. Natl. Acad. Sci. U.S.A.* 91:2679 (1994)], a cAMP sensor protein (called "CAMPS") was identified and it was found that the protein has two putative cAMP binding domains, a Pleckstrin homology domain (PH domain), and a guanine nucleotide exchange factor (GEF) homology domain.

In the course of this study, two study groups independently reported cAMP binding proteins that activate Rap1, a member of the small G binding proteins [J. de Rooij et al. *Nature* 396:474 (1998); H. Kawasaki et al. *Science* 282:2275 (1998)], and CAMPS was incidentally revealed to be a mouse homologue of cAMP-GEFII [H. Kawasaki et al. *Science* 282:2275 (1998)].

Though the mechanisms of intracellular vesicle transport system have thus gradually been clarified, substantial part of them remains still unknown. Further progress is needed for the understanding of the mechanisms so as to provide diagnostic agents or therapeutics for a variety of diseases which involve neurons or endocrine cells.

Unlike the former suggestion that only a single cAMP binding domain was present in cAMP-GEFII, the study by the present inventors suggested the presence of two putative cAMP binding domains (cAMP-A and cAMP-B), based on a sequence alignment of cAMP-GEFII sequence and regulatory subunits of PKA. Figure 1 shows the sequence alignment of the cAMP binding domains. The cAMP binding domains A and B (cAMP-A and cAMP-B, respectively) of cAMP-GEFII and the cAMP binding domains A and B of the PKA regulatory subunit  $I\alpha$  ( $RI\alpha$ -A and  $RI\alpha$ -B, respectively) are shown. The invariant residues in the different cAMP-binding domains are indicated by black boxes.

As shown in Figure 2, a glutathione-S-transferase (GST)-cAMP-A fusion protein bound to [ $^3H$ ]cAMP with a dissociation constant ( $K_d$ ) of  $\sim 10 \mu M$ , while the binding of [ $^3H$ ]cAMP to a GST-cAMP-B fusion protein was not evident under the same conditions.

Figure 2 shows the binding of cAMP to cAMP-A. GST-cAMP-A (filled circles) or GST-PKA  $RI\alpha$  (open circles) was incubated with different concentrations

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of [<sup>3</sup>H]cAMP (0-50 μM). The data for cAMP-A or PKA RI α are normalized relative to maximal cAMP binding activities. K<sub>d</sub> values are 10.0±2.3 μM and 23.7±0.6 nM for cAMP and PKA RI α, respectively.

In the cAMP-B domain, the amino acid residue 423, which originally is glutamic acid (Glu), is substituted with lysine (Lys). This glutamic acid residue is important for cAMP binding. Considering that a more rapid dissociation than the wild-type was observed with a PKA regulatory subunit having an equivalent mutation (E-200-K), cAMP-B may also dissociate cAMP rapidly. Thus, a possibility remains that cAMP binds to the cAMP-B domain.

#### SUMMARY OF THE INVENTION

As identification of a target molecule of CAMPUS, cAMP-GEFII, would serve to show its physiological role, the present inventors attempted to find a molecule that interacts with cAMP-GEFII by means of a yeast two-hybrid screen (YTH) method on the MIN6 cDNA library (See "Identification of Interacting molecules by YTH Method").

Surprisingly, the present inventors found that cAMP-GEFII interacts with a novel isoform (named "Rim2" by the present inventors) of Rim (a molecule which specifically interacts with Rab3: Rab3-interacting molecule: Hereinafter referred to as "Rim1"). Rim1 protein is a putative effector of the small G protein Rab3 and is proposed to serve as a Rab3-dependent regulator of synaptic vesicle fusion [Y. Wang et al. Nature 388:593(1997)].

The full-length novel protein Rim2 sequenced by the present inventors, which consists of 1590 amino acid residues, was found to have 61.6 % identity with rat Rim1. As Figure 3 shows, a zinc finger, PDZ and two C2 domains were found highly conserved between Rim1 and Rim2.

Based on the above findings, the present invention provides a protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing.

The present invention further provides a protein having an amino acid sequence with one or more amino acids deleted, substituted, inserted or added relative to the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing and which has a property to interact with GDP/GTP exchange factor II.

The present invention further provides a mouse gene which encodes the

following proteins (1) or (2):

(1) a protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing,

(2) a protein having an amino acid sequence with one or more amino acids deleted,  
5 substituted, inserted or added relative to the above-identified amino acid sequence and which has a property to interact with GDP/GTP exchange factor II.

In the present specification, "one or more" amino acid residues are generally several (e.g., 3 or 4) to 10 residues.

The present invention further provides a DNA having a nucleotide sequence  
10 set forth under SEQ ID NO:2 in the Sequence Listing, the DNA being a cDNA corresponding to the above protein having the amino acid sequence set forth under SEQ ID NO:1 in the Sequence Listing.

The present invention further provides a DNA having a nucleotide sequence with one or more nucleotides deleted, substituted, inserted or added relative to the  
15 nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing and encoding any one of the above proteins. Herein, "one or more" nucleotides are generally several (e.g., 3 or 4) to 10 nucleotides. A variety of such nucleotide sequences with one or more nucleotides deleted, substituted, inserted or added can be readily prepared by those skilled in the art by making use of the familiar  
20 knowledge on degeneracy of the genetic code.

The present invention further provides a DNA having the nucleotide sequence of the coding region of the any one of the above DNA's or of a DNA having the nucleotide sequence set forth under SEQ ID NO:2 in the Sequence Listing.

The present invention further provides a DNA fragment consisting of a part  
25 of any one of the above DNA's.

The present invention further provides a probe comprising a DNA which hybridizes with the DNA consisting of any one of the above nucleotide sequences.

The present invention further provides a primer DNA fragment consisting of a partial sequence of any one of the above nucleotide sequences.

30 The present invention further provides a recombinant vector having any one of the above DNA's.

The present invention further provides a monoclonal or polyclonal antibody directed to any one of the above proteins.

The present invention further provides a diagnostic agent for human use

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comprising any one of the above probes or antibodies. The diagnostic agent is useful in the test for such diseases as secretion disorders in secretory systems including pituitary, hypothalamus, pancreatic  $\beta$ -cells and parotid gland, or the test for brain-nervous system diseases.

- 5           The present invention further provides a therapeutic agent for any one of the above diseases.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a sequence alignment of the cAMP binding domains.

- 10           Figure 2 is a graph showing the binding of cAMP to cAMP-A.

Figure 3 illustrates a comparison of amino acid identity between Rim1 and Rim2, in zinc finger, PDZ and C2 domains.

Figure 4 shows the results of immunoblotting showing the interaction between cAMP-GEFII and Rim1 or Rim2.

- 15           Figure 5 shows the results of Northern blot analysis of cAMP-GEFII, Rim1 and Rim2 in various rat tissues and endocrine- and neuroendocrine-derived cell lines.

Figure 6 is the result of *In situ* hybridization showing the localization of Rim1 and Rim2 in mouse brain and pituitary.

- 20           Figure 7 is a graph showing the result of yeast two-hybrid assays.

Figure 8 illustrates the result of immunoblotting showing the interaction between Rab3A and Rim1 or Rim2 *in vitro*.

Figure 9 is a graph showing the time course for high  $K^+$ -induced GH secretion from PC12 cells cotransfected with GH and cAMP-GEFII.

- 25           Figure 10 is a graph showing the effect of forskolin on GH secretion from transfected PC cells.

Figure 11 is a graph showing forskolin-induced GH secretion from PC12 cells transfected with various mutant cAMP-GEFII.

- 30           Figure 12 is a graph showing the effect of H-89 on forskolin-induced GH secretion from PC12 cells transfected with cAMP-GEFII.

Figure 13 is a schematic illustration showing a model for cAMP-dependent exocytosis.

#### DETAILED DESCRIPTION OF THE INVENTION

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A variety of mutants can be provided by means of recombinant DNA technology. First, mutations can be introduced into a DNA clone fragment through different chemical and/or enzymatic processes, and the mutant DNA's thus obtained are then sequenced to select particular mutants with intended merits. This method allows a systematic preparation of different mutants regardless of their phenotypes. General methods of preparing a mutant clone DNA are as follows.

1. With the help of an oligonucleotide, substitution, deletion, insertion or addition can be directly effected in a given DNA sequence. This method enables to introduce a number of mutations in a small region of a given DNA.

2. By using longer oligonucleotides, it is possible to synthesize a desired gene.

3. By means of region-specific mutagenesis, a desired mutation can be introduced into a large (1-3 kb) DNA region.

4. Linker-scanning mutagenesis of DNA is a method suited for introducing a cluster point mutation into a relatively small (4-10 bp) DNA region.

5. PCR is also utilized as a method for direct introduction of a mutation.

[References: Current Protocols in Molecular Biology., 3 Vols., Edited by Ausubel F.M. et al., John Wiley & Sons, Inc., Current Protocols., Vol. 1, Chapter 8: Mutagenesis of Cloned DNA, pages 8.0.1-8.5.10]

Also well known to those skilled in the art are methods of preparing plasmids or vectors which can express a desired gene including different mutations obtained by the above methods. That is, by inserting a DNA carrying a desired gene into a expression vector DNA using a combination of restriction enzymes and a ligase, a recombinant plasmid is readily constructed which carries the desired gene. The recombinant plasmid thus obtained is then introduced into different cells to transfect them, thereby producing transformed cells. Cells which may be utilized range from prokaryotes, e.g. *E. coli*, to yeast, insect, plant and animal cells.

[References: Vectors Essential Data. Gacesa P. and Ramji D.P., 166 pages. BIOS Scientific Publishers Limited 1994., John Wiley & Sons in association with BIOS Scientific Publishers Ltd. Expression vectors, pages 9-12.]

Introduction of a recombinant plasmid into host cells is effected by calcium chloride method or electroporation. Calcium chloride method provides efficient transformation without requiring any special apparatus. For higher efficiency,

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electroporation is recommended.

[References: Current Protocols in Molecular Biology, 3 Vols. Edited by Ausbel F.M. et al., John Wiley & Sons, Inc., Current Protocols, Vol. 1, unit 1.8: Introduction of Plasmid DNA into Cells, pages 1.8.1-1.8.10]

5 Two types are known of transfection generally carried out on animal cell lines, i.e., transient and permanent types. In transient transfection, transformed cells are cultured for 1 - 4 days to effect transcription and replication of the transfected gene, and then the cells are harvested and their DNA analyzed. Alternatively, in many studies, a stable transformant cell line is produced, in which  
10 the transfected gene is incorporated into the chromosomes. Examples of the method for transfection include calcium phosphate method, electroporation, and liposome fusion method.

[Reference: Current protocols in molecular biology. 3 vols. Edited by Ausubel F.M. et al., John Wiley & Son, Inc., Current Protocols. Vol. 1, chapter 9: Introduction of  
15 DNA into mammalian cells, pages 9.0.1-9.17.3.]

Polyclonal and monoclonal antibodies directed to the proteins (polypeptides) coded by Rim2 gene of the present invention or their fragments and analogues as well, are readily prepared using techniques well known in the art. Antibodies obtained may be used as laboratory reagents and diagnostic agents for  
20 diseases associated with Rim2 gene. The antibodies obtained are also used for preparation of antibody columns, for immunoprecipitation as well as for identification of the antigen by Western blotting.

A general method for preparing a monoclonal antibody in mg-scale directed to the proteins coded for by Rim2 gene of the present invention is as follows: Mice  
25 are inoculated with the antigen protein to immunize. The spleen is removed from the mice exhibiting a sufficient antibody titer. The spleen cells are dissociated, and selected B cells are fused with myeloma cells of B cell origin to form hybridoma cells which secrete the antibody. The monoclonal antibody secreted from the hybridoma cells is purified from the culture medium using an affinity column, ion-  
30 exchange, or gel filtration, etc. The polyclonal antibody of the present invention may be prepared by a conventional method: Using rabbits, horses, mice or guinea pigs as immunized animals, the antigen protein is inoculated along one of the schedules known in the art to immunize the animals, and then IgG, etc. are isolated from the collected serum.

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[Reference: Current protocols in molecular biology, 3 vols. Edited by Ausubel F.M. et al., John Wiley & Sons, Inc., Current Protocols, Vol. 2, chapter 11: Immunology, pages 11.0.1-11.16.13.]

In order to assess the specificity of interaction between cAMP-GEFII and Rim2, the present inventors evaluated the binding of a FLAG-tagged cAMP-GEFII protein to a GST-Rim2 fusion protein immobilized on glutathione beads (See "Study on Interaction between Rim2 and cAMP-GEFII: I").

Briefly, lysates from COS-1 cells transfected with FLAG-tagged cAMP-GEFII, from MIN6 cells or from mouse brain homogenate were evaluated for binding to GST-Rim1, GST-Rim2 or GST alone. cAMP was detected by immunoblotting with an anti-FLAG antibody (Figure 4, left) or an anti-cAMP-GEFII antibody (Figure 4, center and right), respectively. These results demonstrates that cAMP-GEFII protein interacts with GST-Rim2 protein. Likewise, GST-Rim1 protein also bound to cAMP-GEFII in the mouse brain homogenate (See "Study on Interaction between Rim1 and cAMP-GEFII") (Figure 4, right). These results confirms that cAMP-GEFII interacts with Rim1 and Rim2.

Figure 5 shows the results of the northern blot analyses of cAMP-GEFII, Rim1 and Rim2 in various rat tissues and endocrine- and neuroendocrine-derived cell lines (See "Northern Blotting in Rat Tissues"). Ten  $\mu$ g samples of total RNA from various tissues and cell lines (except 5  $\mu$ g for pancreatic islets) were used. Hybridization and washing were performed under standard conditions. The faint signals seen in Rim2 mRNA blot analysis of cerebrum and cerebellum are due to cross-hybridization with the Rim1 cDNA probe used. Figure 5 shows that Rim2 mRNA is expressed predominantly in endocrine tissues and endocrine- and neuroendocrine-derived cell lines, including pituitary, pancreatic Langerhans' islet cells, MIN6 cells, and PC12 cells. Rim2 mRNA was detected in the brain by reverse transcriptase-PCR (data not shown). Rim1 mRNA, in contrast, was found to be expressed in cerebrum, cerebellum, and pituitary by a similar analysis.

The major transcripts for Rim1 and Rim2 have 6.4 kb for Rim1, and 7.2 kb and 5.4 kb for Rim2. There are also found several minor transcripts, which occur due probably to alternative splicing.

cAMP-GEFII mRNA is generally coexpressed with Rim1 or Rim2 mRNA in tissues and cell lines in which regulated exocytosis is known to occur. Figure 6 illustrates the results of *in situ* hybridization showing the localization of Rim1 and

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Rim2 in mouse brain and pituitary. In the figure: (a) cAMP-GEFII; (b) Rim1; (c) Rim2; (d) pituitary. The scale bar corresponds to 1 mm. Abbreviations: Cb = cerebellum, Cp = caudoputamen, Cx = cortex, Hi = hippocampus, Ob = olfactory bulb, Po = pons, Th = thalamus

5 Rim2 mRNA is found expressed only in the cerebellar cortex, while Rim1 mRNA is expressed in cerebral cortex, hippocampus (especially CA3 and dentate gyrus), olfactory bulb, and cerebellar cortex (See "*In situ* Hybridization in Mouse Brain"). The distribution of cAMP-GEFII mRNA overlaps largely with that of Rim1 mRNA in the brain. It is confirmed that Rim2 mRNA and cAMP-GEFII mRNA are  
10 coexpressed in anterior pituitary.

Rim1 is proposed to be a Rab3 effector, a low molecular weight G protein [Y. Wang, et al., Nature 388,593 (1997)]. Using yeast two-hybrid assays (See "Study on Interaction between Rim2 and Rab3A".), the present inventors found that Rim2, like Rim1, interacts with active Rab3A (Q81L) (Figure 7). Figure 7 shows the  
15 results of the yeast two-hybrid assays. Rim1, Rim2 or rabphilin3 and wild-type Rab3A or constitutively active Rab3A (Q81L) in various combinations were determined by transactivation of liquid  $\beta$ -galactosidase activity.

In addition, the immobilized GST-Rim2 bound only to the GTP $\gamma$ S-bound form of Rab3A (Figure 8). Figure 8 shows the interaction between Rab3A and  
20 Rim1 or Rim2 in vitro, which is the result obtained by incubating GTP $\gamma$ S- or GDP $\gamma$ S-bound form of Rab3A with GST-Rim1 (residues 1-201) and GST-Rim2 (residues 1-345) immobilized on glutathione beads, respectively. Rab3A was detected by immunoblotting with anti-Rab3A antibody. These results indicate that Rim2, like Rim1, binds to the GTP-activated form of Rab3A.

25 The interaction of cAMP-GEFII and Rim2 protein strongly suggests that cAMP-GEFII is involved in regulated exocytosis. To determine its functional role, the present inventors examined the effect of cAMP on Ca<sup>2+</sup>-dependent secretion in PC12 cells cotransfected with growth hormone (GH) and cAMP-GEFII (See "Study on GH secretion from Transfected PC12 Cells").

30 Since PC12 cells endogenously express Rim2 but not cAMP-GEFII, the exogenously introduced cAMP-GEFII may form a complex with endogenous Rim2.

Figure 9 is a graph showing the time course of high K<sup>+</sup>-induced GH secretion from PC12 cells cotransfected with GH and cAMP-GEFII. Figure 10 is a graph showing the effect of forskolin on GH secretion from the transfected PC12

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cells. Forskolin (50  $\mu$ M) was added 10 min before the incubation with a low  $K^+$  (4.7 mM) or high  $K^+$  (60 mM) solution. The meaning of the symbols are as follows: For basal (low  $K^+$ -induced) secretion: cAMP-GEFII-transfectant (filled triangles);  $\beta$ -galactosidase-transfectant (control)(open circles); high  $K^+$ -induced secretion: cAMP-GEFII-transfectant (filled circles);  $\beta$ -galactosidase-transfectant (control)(open circles). The values represent the percent GH amounts released into the medium relative to the total cellular GH amounts.

In the cotransfected PC12 cells, as shown in Figure 9, cAMP-GEFII did not alter  $Ca^{2+}$ -dependent (60 mM  $K^+$ ) secretion of cotransfected GH, compared to the control, but significantly enhanced forskolin (50  $\mu$ M)-induced,  $Ca^{2+}$ -dependent GH secretion (Figure 10). Forskolin acts mainly on adenylate cyclase, serving to increase cAMP levels in the cells. cAMP-GEFII also enhanced 8-Br-cAMP (1 mM)-induced,  $Ca^{2+}$ -dependent GH secretion (cAMP-GEFII-transfectant,  $34.9 \pm 1.3$  %; control,  $25.1 \pm 1.8$  %,  $n=9$ ,  $P<0.001$ ).

Figure 11 is a graph showing forskolin-induced GH secretion from PC12 cells transfected with various mutant cAMP-GEFII, in which the increment of forskolin (50  $\mu$ M)-induced GH secretion (in the presence of high  $K^+$ ) above the basal level during a 15-min incubation for each mutant cAMP-GEFII is expressed as percentage relative to the wild-type cAMP-GEFII (100%). In the figure: WT = wild-type cAMP-GEFII, T810A = mutant cAMP-GEFII (T810A); G114E, G422D = double mutant cAMP-GEFII (G114, G422D).

The forskolin-induced GH secretion was not affected in the mutant cAMP-GEFII (T810A) in which a potential PKA phosphorylation site is disrupted by substitution of one of its amino acids (Figure 11). In addition, the forskolin-induced GH secretion in the mutant cAMP-GEFII (G114E, G422D) in which both of the cAMP binding sites are disputed was reduced to ~40 % of that in the wild-type.

These results indicate that cAMP promotes  $Ca^{2+}$ -dependent GH secretion by binding to cAMP-GEFII, without involving its phosphorylation by PKA.

Figure 12 is a graph showing the effect of H-89 on forskolin-induced GH secretion from cAMP-GEFII-transfected PC12 cells. H-89 (10  $\mu$ M) was added to the incubation buffer 10 min before forskolin (50  $\mu$ M) treatment. The treatment with H-89 (10  $\mu$ M) reduced high  $K^+$ -induced GH secretion in both of the cAMP-GEFII-transfected and  $\beta$ -galactosidase-transfected PC12 cells. The data were obtained from 3-5 independent experiments (A-D). The values are means  $\pm$  SEM

(P<0.01).

Importantly, the forskolin-induced,  $\text{Ca}^{2+}$ -dependent GH secretion from the cAMP-GEFII-transfected PC12 cells treated with the PKA inhibitor H-89 was significantly higher than that from the control cells. This indicates that cAMP-GEFII mediates cAMP-dependent and PKA-independent exocytosis.

To ascertain the physiological relevance of cAMP-GEFII, the present inventors investigated the role of endogenous cAMP-GEFII in secretion. In insulin secretion from pancreatic  $\beta$ -cells, cAMP is proposed to stimulate exocytosis by PKA-dependent as well as PKA-independent mechanisms [M. Prentki, F.M. Matschinsky, *Physiol. Rev.* 67:1185 (1987)/ P.M.Jones, S.J. Persaud, *Endocrine. Rev.* 19:429 (1998)].

In the high glucose condition of 16.7 mM, 8-Br-cAMP-induced insulin secretion from MIN6 cells treated with antisense oligonucleotides against cAMP-GEFII was significantly reduced ( $87.5 \pm 2.3$  % of the secretion from MIN6 cells treated with a control oligonucleotide,  $n=27$ ,  $P<0.005$ ) (See "Study of the Role of cAMP-GEFII in cAMP-dependent Exocytosis"), suggesting that cAMP-GEFII participates in cAMP-dependent exocytosis in native cells.

Rab3 is associated with the final step of exocytosis. The structurally-related proteins rabphilin3 [H. Shirataki et al., *Mol. Cell. Biol.* 13,2061 (1993)] and Rim1 both bind to Rab3A, suggesting that multiple Rab3A effectors could operate in triggering docking and fusion of the vesicles to the plasma membrane.

In the process toward the present invention, it was found that the cAMP sensor, cAMP-GEFII, mediates cAMP-induced,  $\text{Ca}^{2+}$ -dependent exocytosis by interacting with a Rab3 effector Rim2.

In addition to its role in PKA phosphorylation of proteins associated with secretory processes, previous studies have suggested that cAMP may act directly on the exocytosis [G. Lonart, et al., *Neuron* 21:1141 (1998); E. Renstrom, et al., *J. Physiol.* 502:105(1997); K. Yoshimura et al., *Biochim. Biophys. Acta* 1402:171(1998)]. In pancreatic  $\beta$ -cells, too, PKA-dependent as well as PKA-independent stimulation of insulin release by cAMP has been proposed [E. Renstrom, et al., *J. Physiol.* 502:105 (1997)]. It is thought that cAMP probably directly stimulates amylase release in parotid acinar cells [G. Lonart, et al., *Neuron* 21:1141 (1998)]. In addition, a recent study suggests that cAMP enhances glutamate release in the brain partly by a direct action on the exocytotic machinery

[G. Lonart, et al., Neuron 21,1141 (1998)].

However, while both rabphilin3 and Rim1 are ubiquitously expressed in most of the synapses in the brain[C. Li et al., Neuron 13:885 (1994)], cAMP-enhanced glutamate release occurs in synaptosomes from the CA3 region in the hippocampus, not from the CA1 region, a finding consistent with cAMP-GEFII and Rim1 being coexpressed predominantly in CA3.

Accordingly, it is considered that, in addition to PKA-dependent phosphorylation in the secretory processes, cAMP promotes regulated exocytosis in a PKA-independent manner by acting directly on a complex of cAMP-GEFII (a cAMP sensor) and Rim (a Rab3 effector) in some neurons and neuroendocrine and endocrine cells, as schematically illustrated in Figure 13.

These findings indicates that Rim2 of the present invention also plays an important role in the regulation of exocytosis in neurons and endocrine cells.

## 15 EXAMPLES

The present invention will be described below in further detail by presenting specific procedures in the present invention with reference to an example.

### <Sequencing of CAMPS (cAMP-GEFII) cDNA>

20 A plasmid cDNA library has been made from a mouse insulin-secreting cell line, MIN6, in the vector pVP16. A yeast two-hybrid bait vector was constructed in plasmid pBTM116 using a DNA fragment encoding partial rat SUR1 (amino acid residues 598-1003)(GenBank accession number L40624), a subunit of the pancreatic  $\beta$ -cell  $K_{ATP}$  channel.

25 Yeast two-hybrid screen of the plasmid MIN6 cDNA library was performed as described in K. Kotake et al., J. Biol. Chem. 272:29407 (1997). A prey clone encoding a partial CAMPS, a cAMP sensor, (residues 187-730) was isolated. A full-length mouse CAMPS cDNA was obtained from the  $\lambda$  MIN6 cDNA library [N. Inagaki et al., Proc. Natl. Acad. Sci. U.S.A. 91:2679(1994)]. The nucleotide  
30 sequence of mouse CAMPS (cAMP-GEFII) has been deposited in Genbank with the accession number of AB021132.

### <Preparation and Test of GST fusion Protein>

cAMP-A (amino acid residues 43-153), cAMP-B (amino acid residues 357-469), and rat PKA regulatory subunit (RI $\alpha$ )(full-length) were expressed as GST-

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fusion proteins using pGEX-4T-1 (Amersham-Pharmacia) and purified according to the manufacturer's instructions. cAMP binding assay was performed as described in R.A. Steiberg, et al., J. Biol. Chem. 262:2664(1987) with slight modifications.

5 Briefly, GST-fusion protein (1  $\mu$ g) was incubated in binding buffer (200  $\mu$ l) containing various concentrations of [ $^3$ H]cAMP, 50 mM potassium phosphate buffer (pH 6.8), 150 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.5 mg/ml bovine serum albumin with or without 40 mM unlabeled cAMP for 2 hrs on ice.

#### <Identification of Interacting molecules by YTH Method>

10 Yeast two-hybrid bait vector was constructed in plasmid pBTM116 using a full-length mouse cAMP-GEFII cDNA. A prey clone encoding a partial sequence of Rim2 (amino acid residues 53-863) was isolated from the plasmid MIN6 cDNA library. A full-length cDNA for Rim2 was obtained from the  $\lambda$  MIN6 cDNA library.

#### <Study on Interaction between Rim2 and cAMP-GEFII: I>

15 Rim2 (amino acid residues 538-863) was expressed as a GST fusion protein and purified according to the method described in "Preparation and Test of GST fusion Protein". A full-length cAMP-GEFII cDNA was subcloned into plasmid pFLAG-CMV-2 (Sigma). The resultant construct was transfected into COS-1 cells, using LipofectAMINE (Life Technologies). The lysate of the COS-1 cells was  
20 incubated with GST-Rim2 immobilized on glutathione beads for 2 hrs at 4°C. The complex thus obtained was washed with distilled water, separated by SDS-PAGE, and immunoblotted with an anti-FLAG M2 antibody (Sigma).

#### <Study on Interaction between Rim2 and cAMP-GEFII: II>

The lysate of MIN6 cells was incubated with GST-Rim2 and interaction  
25 between cAMP-GEFII and Rim2 was evaluated according to the method described in "Study on Interaction between Rim2 and cAMP-GEFII: I", using a IgG antibody raised against the C-terminus (amino acid residues 1001-1011, Gln-Met-Ser-His-Arg-Leu-Glu-Pro-Arg-Arg-Pro) (SEQ ID NO:5) of mouse cAMP-GEFII.

#### <Study on Interaction between Rim1 and cAMP-GEFII>

30 According to the method described in "Preparation and Test of GST fusion Protein", Rim1 partial sequence (530-806) was expressed as a GST fusion protein and then purified. The brain homogenate from three mice was incubated with GST-Rim1 immobilized on glutathione beads overnight at 4°C. cAMP-GEFII was detected as described in "Study on Interaction between Rim2 and cAMP-GEFII: II".

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<Northern Blotting in Rat Tissues>

Northern Blotting was performed for various tissues of rat using, as probes, mouse cAMP-GEFII (nucleic acids 606-2237), rat Rim1 (1035-1491), and mouse Rim2 (586-1490) cDNA.

5 <In situ Hybridization in Mouse Brain>

*In situ* hybridization in mouse brain was performed as described in J. Tanaka, M. Murate, C.Z. Wang, S. Seino, T. Iwanaga, Arch. Histol. Cytol. 59:485 (1996).

10 Antisense oligonucleotide probes (45 mer) used for mouse cAMP-GEFII and Rim2 correspond to the regions of the nucleic acids 2746-2790 and 1376-1420, respectively.

For the antisense oligonucleotide for Rim1, Rim1 cDNA was partially cloned from mouse brain: the probe used in this was 5'-ttgcgctcactcttctggcctcccttgccattctgctctgaaagc-3' (SEQ ID NO:3).

15 <Study on Interaction between Rim2 and Rab3A>

According to the method described in "Identification of Interacting molecules by YTH Method", the full-length cDNA's for wild type mouse Rab3A and constitutively active bovine Rab3A (Q81L) were cloned into the yeast bait vector pBTM116.

20 The nucleotide sequence of zinc finger domains of bovine rabphilin3 (amino acid residues 1-283), rat Rim1 (amino acid residues 1-204) and mouse Rim2 (amino acid residues 1-345) were cloned into the prey vector pVP16. Liquid culture assay of  $\beta$ -galactosidase activities was performed according to the manufacturer's instructions (Clontech). The activity values were obtained from 3 independent clones for each transformant and normalized by cell numbers determined as OD<sub>600</sub>.

25 Lipid-modified Rab3A was purified from the membrane fraction of Sf9 cells expressing Rab3A. Rat Rim1 (amino acid residues 1-204) and mouse Rim2 (amino acid residues 1-345) were expressed as GST fusion proteins and purified. The GTP  $\gamma$  S- or GDP  $\beta$  S-bound form of Rab3A was incubated for 90 min at 4°C with GST-Rim1, or GST-Rim2 (30 pmol for each) immobilized on glutathione beads in reaction buffer. Rab3A was detected by immunoblotting with anti-Rab3A antibody.

30 <Study on GH secretion from Transfected PC12 Cells>

GH secretion from transfected PC12 cells was performed as described in K. Korake et al., J. Biol. Chem., 272:29407(1997). Expression plasmid vectors (pSR

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$\alpha$ ) for wild-type cAMP-GEFII, mutant cAMP-GEFII (T810A), and the double mutant cAMP-GEFII (G114E, G422D) were prepared. As a control,  $\beta$ -galactosidase ( $\beta$ -gal) was used. PC cells were transfected with GH expression vector (pXGH5: Nichols Institute) plus each vector described above, using LipofectAMINE.

5        PC12 cell were incubated with a low  $K^+$  (4.7 mM) or high  $K^+$  (60 mM) solution, in the presence or absence of forskolin (50  $\mu$ M) or 8-bromoadenosine 3',5' cyclic monophosphate (8-Br-cAMP)(1 mM). Forskolin or 8-Br-cAMP was added 10 min before the incubation with a low or high  $K^+$  solution. In some experiments, the PKA inhibitor H-89 (10  $\mu$ M) was added 10 min before forskolin stimulation.

<Study of the Role of cAMP-GEFII in cAMP-dependent Exocytosis>

To interfere with the synthesis of cAMP-GEFII in MIN6 cells, antisense phosphorothioate-substituted oligoDNA (16 mer) against mouse cAMP-GEFII (the region corresponding to nucleic acids 104-119) and control oligoDNA (5'-acctacgtgactacgt-3') (SEQ ID NO:4) were synthesized (BIOGNOSTIK).

15        MIN6 cells were treated with 4  $\mu$ M of the antisense oligoDNA or control oligoDNA 24 hours before insulin secretion experiments. The efficacy of antisense oligoDNA was evaluated by immunoblot analysis of the antisense oligoDNA-treated MIN6 cells over-expressing cAMP-GEFII by transient transfection, using anti-cAMP-GEFII antibody. The level of cAMP-GEFII was markedly lowered in the antisense oligoDNA-treated MIN6 cells. Insulin secretory response to 8-Br-cAMP (1 mM) of these MIN6 cells was assessed in the presence of high glucose (16.7 mM). Five separate experiments were performed, in which insulin was measured as described in T. Gonoï et al., J. Biol. Chem. 269:16989 (1994).

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